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The effect of electron acceptor variations on the behaviour of *Thiosphaera pantotropha* and *Paracoccus denitrificans* in pure and mixed cultures

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1. SUMMARY

The competitive advantages provided by a capacity for aerobic denitrification have been tested by comparing *Thiosphaera pantotropha* (which denitrifies aerobically and anaerobically), with a strain of *Paracoccus denitrificans* (which only denitrifies under anaerobic conditions) in acetate-limited chemostats. A comparison of μ-C_S curves based on K_S and μ_{max} measurements indicated that *Pa. denitrificans* could be expected to dominate mixtures of the two species at high growth rates when the dissolved oxygen was above 80% of air saturation and NH_3 was the sole source of nitrogen. The comparison also suggested that at lower growth rates, lower dissolved oxygen tensions, and/or in the presence of nitrate, *Tsa. pantotropha* should have the competitive advantage. Chemostat experiments with mixtures of the two species showed that *Tsa. pantotropha* did, indeed, dominate the population when expected. However, when *Pa. denitrificans* was expected to dominate, only a small increase in the *Pa. denitrificans* numbers was possible before *Tsa. pantotropha* formed a biofilm on the walls of the chemostat instead of washing out, and was again able to out-compete *Pa. denitrificans* for acetate. Experiments with axenic chemostat cultures subjected to aerobic/anaerobic switches showed that *Tsa. pantotropha*, with its constitutive denitrifying system, was able to adjust smoothly to the changing environmental conditions and thus continued to grow. *Pa. denitrificans* does not have constitutive denitrifying enzymes, and could consequently not adjust its metabolism to the lack of oxygen rapidly enough. It therefore washed out at a rate equivalent to the dilution rate.

2. INTRODUCTION

The occurrence of aerobic denitrification is now well documented (see ref. 1 for a review). *Thiosphaera pantotropha* is among the constitu-

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tive denitrifiers able to denitrify at dissolved oxygen concetrations approaching air saturation. *Tsa. pantotropha* was isolated as one of the dominant organisms in a denitrifying waste-water treatment reactor which was generally anaerobic but received occasional pulses of oxygen [2]. Against this background, and given some of the (eco)physiological properties of *Tsa. pantotropha* and other similar strains, the postulated ecological niche for these organisms appears to lie in situations where oxygen is limiting, or when the environment goes through relatively short cycles of aerobiosis and anaerobiosis [1,3,4]. *Tsa. pantotropha* is, of course, also a heterotrophic nitrifier [5,6,7]. Both nitrification and denitrification are of importance in soil fertility (where they are undesirable) and waste water treatment (where both phenomena play an important part in nitrogen removal). An understanding of the factors which will select for bacteria that can simultaneously nitrify and denitrify is thus desirable. Because of the complexity of experiments to test the combined phenomena and the number of variables involved, it was decided that their ecological importance should be investigated separately. Experiments were therefore set up to discover whether, and under what conditions, its abilities as an aerobic denitrifier would give *Tsa. pantotropha* a selective advantage over *Paracoccus denitrificans*, a species which only denitrifies under anaerobic conditions.

The problem was approached in two ways: firstly, the outcome of competition between the two species in different acetate-limited chemostat cultures was checked; secondly, the effect of aerobic/anaerobic switches on axenic cultures of *Tsa. pantotropha* and *Pa. denitrificans* was measured in order to test their adaptability. This paper reports the results of these experiments.

3. MATERIALS AND METHODS

Organisms

Thiosphaera pantotropha LMD 82.5 was originally isolated from a denitrifying, sulphide oxidizing wastewater treatment system [2]. *Paracoccus denitrificans* LMD 22.21 was obtained from the culture collection of this laboratory, and is the strain isolated by Beijerinck [8].

Media

The medium for the chemostats contained (g l^{-1}): K_2HPO_4, 0.8; KH_2PO_4, 0.3; NH_4Cl, 0.4; $MgSO_4 \cdot 7H_2O$, 0.4: and 2 ml of trace element solution. Sodium acetate (20 mM) was supplied as the limiting substrate. When appropriate, 32 mM KNO_3 was used.

The medium described for the growth of *Thiobacillus versutus* (formerly *Thiobacillus* A2) by Taylor and Hoare [9] was used for batch cultures. It contained (in g l^{-1}): $Na_2HPO_4 \cdot 7H_2O$, 7.9; KH_2PO_4, 1.5; NH_4Cl, 0.3; $MgSO_4 \cdot 7H_2O$, 0.1; 2ml of trace element solution. The $MgSO_4$, trace element solution, substrates and KNO_3 (20 mM) were added after sterilization. The initial concentration of the sodium acetate in the batch cultures was 10 mM.

The trace element solution [10] used with all media contained (as g l^{-1}): EDTA, 50; $ZnSO_4$, 2.2; $CaCl_2$, 5.5; $MnCl_2 \cdot 4H_2O$, 5.06; $FeSO_4 \cdot 7H_2O$, 5.0; $(NH_4)\ 6Mo_7O_24 \cdot 4H_2O$, 1.1; $CuSO_4 \cdot 5H_2O$, 1.57; $CoCl_2 \cdot 6H_2O$, 1.61.

For colony counting, 2% (w/v) Difco bacto agar was added to the batch culture medium. The substrate in these plates was a mixture of 0.3 g l^{-1} yeast extract, 0.1 g l^{-1} fructose and 0.1 g^{-1} sodium acetate. As *Tsa. pantotropha* is more sensitive to ampicillin than *Pa. denitrificans*, plates with and without 0.15 mg l^{-1} ampicillin were used to obtain *Pa. denitrificans* and total counts, respectively.

Continuous cultures

Continuous cultures were made in chemostats fitted with dissolved oxygen and pH control. The temperature was maintained at 30°C and the pH at 8.0.

For the competition experiments, *Tsa. pantotropha* and *Pa. denitrificans* were grown axenically to steady state at the appropriate dilution rate and dissolved oxygen concentration (80% of air unless otherwise specified). Once the steady state had been confirmed by analysis of the medium and biomass, the two cultures were mixed. To avoid potential contamination prob-

lems during the mixing, the chemostats had been linked before sterilization by tubes through which the cultures could be pumped. In a control experiment, it was confirmed that each of the test species grew normally in filter-sterilized medium in which the other had previously been grown, indicating that neither produced excretion products which would be inhibitory to the other.

For the aerobic/anaerobic and anaerobic/aerobic experiments, the aerobic (80% of air saturation) and anaerobic cycles in the cultures were achieved by sparging with air or ultra-pure nitrogen. Cultures were grown to steady state before a switch was achieved by suddenly changing the sparging gas. The behaviour of the cultures was monitored with continuous, on-line optical density measurements and periodic sampling for off-line analysis.

Biomass analysis

Protein was measured spectrophotometrically, by means of the Micro-Biuret method [11]. Total organic carbon was measured using a Beckman Tocamaster Model 150B.

Because *Tsa. pantotropha* tends to make poly-B hydroxybutyrate (PHB) under some growth conditions, both dry weight and total organic carbon measurements could give an artificially high yield. The yield estimates for the nitrogen balances were therefore based on the protein determinations, as previously described [7].

Analysis of culture medium

Acetate was determined with acetyl-coenzyme A synthetase using a test kit (Boehringer).

Nitrite was measured colourimetrically, with the Griess-Romijn reagent [12] or by means of an HPLC fitted with a ionosphor-TMA column (Chrompak) and a Walters RI detector. Nitrate was also measured with the HPLC.

Hydroxylamine and ammonia were determined colourimetrically by means of the methods described by Frear and Burrell [13] and Fawcett and Scott [14], respectively. As, at the pH values used in these experiments, ammonia and ammonium would both be present, the term 'ammonia' will be used throughout to indicate both the protonated and unprotonated forms.

Miscellaneous

The maximum specific growth rates for the two species under each set of growth conditions were determined during the batch culture start-up phase of each chemostat experiment.

Oxygen uptake was measured using a Clark-type electrode mounted in a thermostatically controlled cell which is closed except for a small hole through which additions may be made.

Apparent K_S values for acetate were determined using direct linear plots [15] of the oxygen uptake rate as a function of various acetate concentrations. Washed cells were resuspended in 0.05 M phosphate buffer, pH 8.0, and the measurements were done in the presence or absence of ammonia and nitrate.

Immuno-fluorescent staining was carried out using antibodies raised against *Tsa. pantotropha* by the method described by Muyzer et al.[16].

4. RESULTS AND DISCUSSION

4.1. Competition studies

4.1.1. Theoretical models

The effect of various environmental factors on the outcome of competition between two species can frequently be predicted from the comparison of theoretical plots of the growth rates versus the residual substrate concentration (μ/C_S curves) constructed according to the Monod formula [17]. Examples of such curves, in which the chemostat dilution rate (D) is plotted against C_S (as $D = \mu$ in a chemostat), for *Tsa. pantotropha* and *Pa. denitrificans*, when grown aerobically on acetate in the presence and absence of nitrate, are shown in Fig. 1. From these curves, it appeared that *Pa. denitrificans* should dominate aerobic mixed cultures of the two species at high growth rates when ammonia was the sole source of nitrogen, and *Tsa. pantotropha* should do better at lower dilution rates (Fig. 1a). If nitrate and oxygen were both present in the medium, the increase in the μ_{max} of *Tsa. pantotropha* [3] should confer a selective advantage over *Pa. denitrificans* at all growth rates (Fig. 1b). At lower dissolved oxygen concentrations ($< 25\%$ of air saturation), the μ_{max}

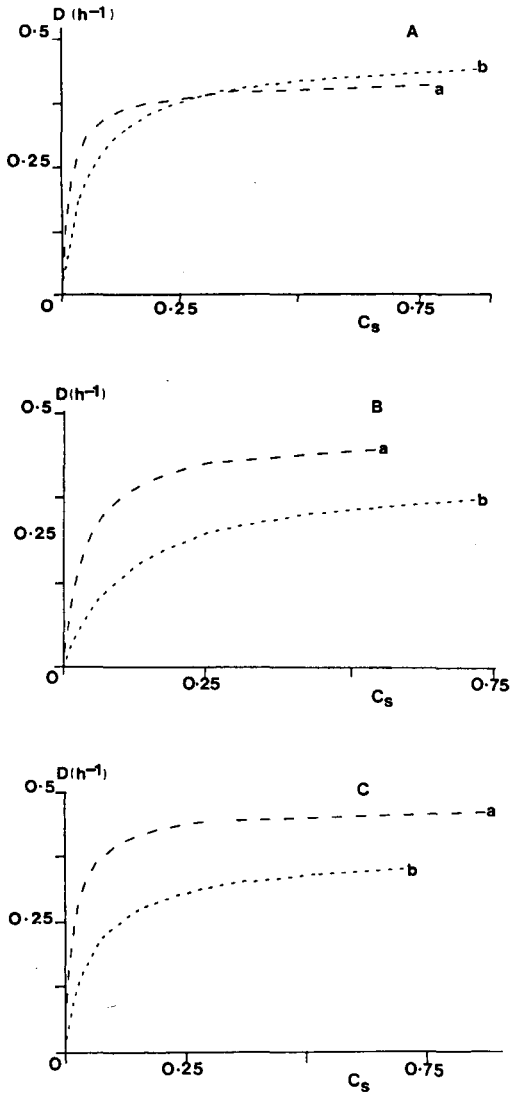

Fig. 1. Curves of dilution rate (D) against residual substrate concentration (C_S) showing the theoretical outcome of competition for acetate between *Tsa. pantotropha* (a) and *Pa. denitrificans* (b) in the chemostat. K_S acetate values of 28 and 63 μM, respectively, were used. 1A = 80% air saturation, NH_3 as sole nitrogen source, $\mu_{max} = 0.42$ and 0.47 h^{-1}, respectively; 1B = 95% air saturation, NH_3 and NO_3^- both supplied, $\mu_{max} = 0.32$ and 0.25h^{-1}, respectively; 1C = 25% air saturation, NH_3 as sole nitrogen source, $\mu_{max} = 0.47$ and 0.38 h^{-1}, respectively.

of *Tsa. pantotropha* increased to such an extent that, even when oxygen was the sole electron acceptor provided, it should have a selective advantage, even at high dilution rates. The position was reversed in anaerobic cultures, where *Pa. denitrificans* had a much higher μ_{max} than *Tsa. pantotropha*, and should therefore dominate at all growth rates (not shown).

4.1.2. Experimental verification

Experimental testing of these models was carried out by competition experiments in the chemostat. The changes in the numbers of cells from the two species were followed after mixing until a steady state was reached. Cell ratios were determined by selective plating and by immunofluorescent labelling (see MATERIALS AND METHODS for details). The experiments gave the predicted results whenever *Tsa. pantotropha* was expected to dominate (Fig. 1a–c), and the cultures remained well suspended and homogenous. In these mixtures, *Pa. denitrificans* amounted to less than 1% of the population after more than 10 volume changes, irrespective of the ratios of the two species in the original culture. Confirmation that the majority population was *Tsa. pantotropha* came from the measurements of the yields and nitrogen balances. For example, as the percentage of *Pa. denitrificans* in the community receiving oxygen as the sole electron acceptor fell from its original 90% value, the biomass concentration also fell. It eventually reached a value similar to that obtained with axenic *Tsa. pantotropha* cultures, that is changing from 8.1 to 8.7 g protein mol acetate^{-1} at the start to 6.2 to 6.6 g protein mol acetate^{-1} at the end. Moreover, the ammonia loss from the axenic *Pa. denitrificans* cultures, which had been very low (equivalent to a nitrification rate of 6 nmol min^{-1} mg protein^{-1} at a dilution rate of 0.07 h^{-1}), rose after mixing to values equivalent to those in the *Tsa. pantotropha* cultures (e.g. 18 nmol^{-1} min^{-1} mg protein^{-1} at a dilution rate of 0.07 h^{-1}).

The results obtained with the cultures in which *Pa. denitrificans* was expected to dominate were less clear cut. For example, when the dilution rate was increased to a value above the cross point of the curves shown in Fig. 1a (and thus at which *Tsa. pantotropha* might be expected to wash out), the population of *Pa. denitrificans* began to recover, increasing from 1% to 13% of

the community after 5 volume changes. Unfortunately, at this point, a rapidly thickening biofilm became apparent on the wall of the chemostats, and the suspended *Pa. denitrificans* population fell back to below 1%. Immunofluorescent staining showed that this biofilm consisted almost entirely of *Tsa. pantotropha*. Of course, *Tsa. pantotropha* was originally isolated from a fluidized bed column where the biomass was attached to sand. One of the main selective pressures in such a system is the ability to attach to surfaces when the dilution rate exceeds the μ_{max} of the organism [18]. As *Tsa. pantotropha* has a much lower K_S for acetate than *Pa. denitrificans* (28 and 63 μM, respectively), it is clear that the attached *Tsa. pantotropha* was able to build a population sufficiently large to successfully compete for substrate even though the *Pa. denitrificans* remained in suspension.

4.2. Aerobic / anaerobic cycles

To test the prediction that *Tsa. pantotropha* (as an aerobic denitrifier) would have an advantage over *Pa. denitrificans* (as a specialist which only denitrifies anaerobically) in situations where the oxygen supply fluctuated, cultures were grown in chemostats undergoing alternate cycles of aerobiosis and anaerobiosis. To allow the study of the behaviour of *Pa. denitrificans* under this regime, without the masking effect caused by potential *Tsa. pantotropha* biofilm formation, the experiments were run with pure cultures.

4.2.1. Aerobic to anaerobic switch

When aerobic cultures were grown to steady state and then suddenly made anaerobic, the nitrate concentrations in both cultures began to fall immediately, but this fall was much more dramatic in the *Pa. denitrificans* cultures (Fig. 2a). While nitrite did not accumulate in the *Tsa. pantotropha* medium (Fig. 2b), its concentration in the *Pa. denitrificans* cultures rose in an almost 1:1 ratio with the decrease in the nitrate, reaching 15 mM after 4 h. A similar, transient (3 h) accumulation of nitrite, accompanied by a fall-off in cytochrome oxidase activity, was observed with batch cultures of *Pa. denitrificans* which had been switched from aerobic to oxygen-limited growth in the presence of nitrate [19].

Regardless of the dilution rate, the protein content of the *Pa. denitrificans* cultures fell substantially during the experiment (by 25% at $D = 0.15\ h^{-1}$, by 50% at $D = 0.30\ h^{-1}$), confirming the wash out indicated by previous optical density measurements (Fig. 3). In contrast, the protein content of the *Tsa. pantotropha* cultures changed very little until the dilution rate was increased to a value above the anaerobic μ_{max}, when the culture washed out. The total organic carbon of all

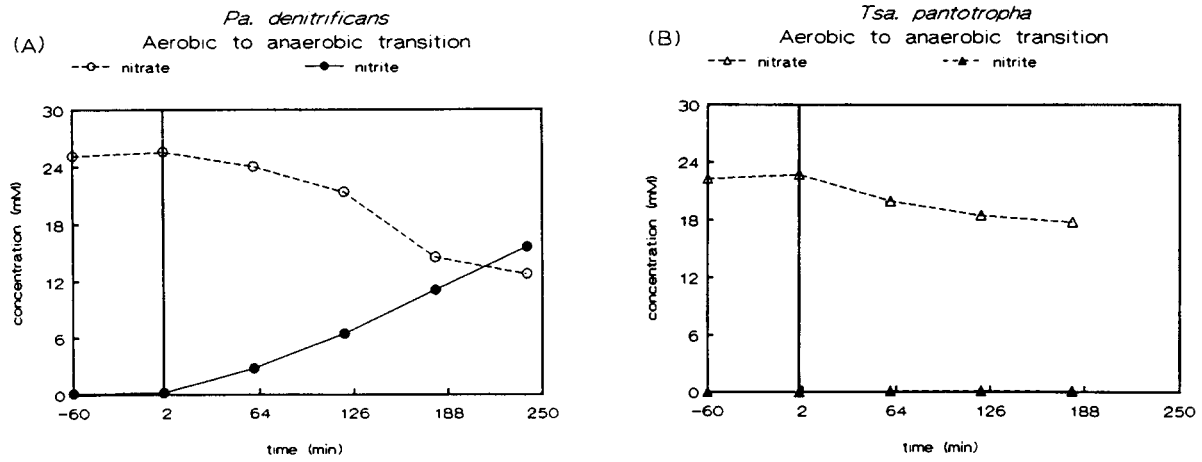

Fig. 2. The response of steady state, acetate-limited, aerobic chemostat cultures ($D = 0.15\ h^{-1}$) of *Pa. denitrificans* (2a) and *Tsa. pantotropha* (2b) to a sudden switch to anaerobiosis, as demonstrated by the changes in the nitrate and nitrite concentrations. The bar indicates the change in the gas stream from air to nitrogen at $t = 0$.

of the supernatents rose after the cultures were made anaerobic, indicating that acetate had ceased to be limiting. Although this was a temporary state of affairs in the *Tsa. pantotropha* cultures, which soon became acetate-limited again, this was not the case in the *Pa. denitrificans* cultures. Electron microscopy revealed the presence of poly-β-hydroxybutyrate in both *Tsa. pantotropha* and *Pa. denitrificans* cells at the end, but not the start of the experiments.

4.2.2. Anaerobic to aerobic switch

Anaerobic cultures of *Tsa. pantotropha* and *Pa. denitrificans* which had been grown to steady state adjusted smoothly when suddenly switched to a dissolved oxygen concentration 80% that of air saturation, confirming the constitutive nature of both aerobic respiratory chains. Immediatedly the air supply was connected, the nitrate concentrations in both cultures began to rise. Neither species accumulated nitrite. The protein content of both cultures increased, confirming the switch to the energetically more efficient use of oxygen as a terminal electron acceptor. Of course, *Tsa. pantotropha* continued to partially respire by means of the denitrification pathway, and its increase in yield was therefore relatively smaller (from 84 mg/l, anaerobically, to 110, aerobically) than that of *Pa. denitrificans* (98 to 138, respectively).

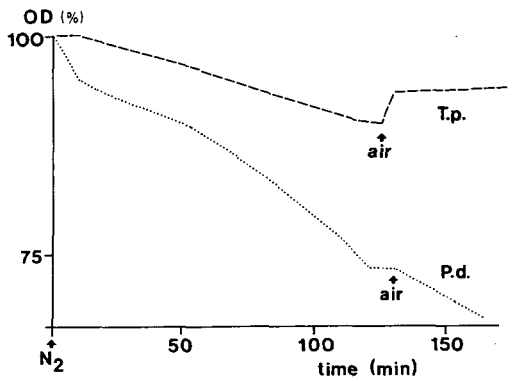

Fig. 3. The outcome of switching steady-state, acetate-limited, chemostat cultures ($D = 0.25\ h^{-1}$) of *Tsa. pantotropha* (T.p.) and *Pa. denitrificans* (P.d.) from aerobiosis (80% air saturation) to anaerobiosis.

Table 1

The effect of nitrite on acetate-dependent oxygen uptake by washed cells of *Tsa. pantotropha* and *Pa. denitrificans* in the presence and absence of ammonia (A: cells grown aerobically in an acetate-limited chemosat in the presence of ammonia and nitrate. B: cells grown aerobically in an acetate-limited chemosat in the presence of ammonia and nitrite.)

Additive		Oxygen uptake rate (nmol O_2 min^{-1} (mg protein)$^{-1}$)		
NH_4^+ (mM)	NO_2^- (mM)	*Tsa. pantotropha*		*Pa. denitrificans*
		A	B	A
0	0	311	336	208
0	2.5	301	349	172
0	5.0	301	343	146
0	10.0	284	334	105
0	15.0	283	336	87
0	20.0	254	nd	nd
7.5	0	284	344	182
7.5	2.5	315	nd	155
7.5	5.0	313	343	126
7.5	10.0	316	349	95
7.5	15.0	nd	334	68
7.5	20.2	293	334	48

4.3. The influence of nitrite

During the earlier experiments shown in Fig. 3, the *Pa. denitrificans* cultures continued to wash out after aerobiosis had been restored. In the light of the results reported above, and because the rate of acetate-dependent oxygen uptake by washed, nitrite-free cells of *Pa. denitrificans* sampled after different periods of anaerobiosis did not change significantly, permanent inactivation of the cytochrome oxidases during anaerobic growth did not seem very likely. It therefore seemed possible that the failure of the culture to recover was due to the level of nitrite in the culture.

Nitrite has previously been shown to inhibit oxygen uptake by *Pa. denitrificans* [5,20], and this also proved to be the case with resting cells from these cultures. The rate of acetate-dependent oxygen uptake by washed *Pa. denitrificans* cells fell as the nitrite concentration increased, while *Tsa. pantotropha* remained relatively unaffected (Table 1). *Tsa. pantotropha* which had been grown in cultures where nitrite replaced the nitrate was completely unaffected by the nitrite concentrations used (Table 1).

Nitrate reductase activity in *Pa. denitrificans* is clearly much less sensitive to oxygen than nitrite reductase (Fig. 2; [21,22]). If oxygen uptake was inhibited to some extent by the high nitrite concentration, the cytochrome chain would become more reduced. Electrons would then be able to flow to nitrate, generating more nitrite and creating a 'vicious circle'. Presumably, if permitted a longer period of anaerobiosis (for example, in batch cultures where the culture density could not fall to too low a level for a viable culture) *Pa. denitrificans* would be able to generate its complete denitrifying pathway and reduce the nitrite concentration.

5. CONCLUSION

As judged by theoretical predictions of the type shown in Fig. 1, the fate of *Tsa. pantotropha* in competition with *Pa. denitrificans* for acetate depends very much on the growth rate, and thus on the available electron acceptors. *Pa. denitrificans* appears to have an advantage if oxygen or nitrate are available as single electron acceptors, especially at higher growth rates. However, in the presence of both electron acceptors, the μ_{max} of *Tsa. pantotropha* becomes greater than that of *Pa. denitrificans* and it will gain a competitive advantage. Another important factor was the concentration of the dissolved oxygen in the cultures. Even when oxygen was the sole electron acceptor, *Tsa. pantotropha* grew faster than *Pa. denitrificans* at low ($< 30\%$ air saturation) oxygen tensions, with the result that it should also dominate mixed cultures under these conditions.

The results of the experimental verification of these models by measuring population changes in communities competing for acetate emphasize the need for caution in the design of experiments of this type. Although the initial experiments appeared to confirm the models, with *Tsa. pantotropha* dominating the communities when expected, it would appear that the ability to adhere to a surface, forming a biofilm, gives a major competitive advantage which outweighs the influence of the growth rate. The models will therefore be tested using another heterotrophic nitrifier/aerobic denitrifier which does not form biofilms so readily.

The response of the two types of denitrifier to a sudden onset of anaerobiosis revealed, as expected, that the possession of a constitutive denitrification pathway can be a selective advantage when sudden changes in the available electron acceptor occur. The advantage gained was twofold: it prevented the build-up of inhibitory amounts of nitrite and it allowed the smooth transition to anoxic growth. It therefore appears that the possession of a constitutive denitrification system provides *Tsa. pantotropha* with a competitive advantage for life under (relatively short-term) oxygen fluctuation, whereas its inability to use more than one electron acceptor at a time makes this *Pa. denitrificans* strain better suited to semi-steady-state conditions.

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REFERENCES

[1] Robertson, L.A. and Kuenen, J.G. (1990) Combined heterotrophic nitrification and aerobic denitrification in *Thiosphaera pantotropha* and other bacteria. Antonie van Leeuwenhoek 57,139–152.

[2] Robertson, L.A. and Kuenen, J.G. (1983) *Thiosphaera pantotropha* gen. nov. sp. nov., a facultatively anaerobic, facultatively autotrophic sulphur bacterium. J. Gen. Microbiol. 129,2847–2855.

[3] Robertson, L.A. and Kuenen, J.G. (1984) Aerobic denitrification: a controversy revived. Arch. Microbiol. 139,351–354.

[4] Robertson, L.A. and Kuenen, J.G. (1984) Aerobic denitrification - old wine in new bottles? Ant. van Leeuwenhoek 50,525–544.

[5] Robertson, L.A. and Kuenen, J.G. (1988) Heterotrophic nitrification in *Thiosphaera pantotropha*: oxygen uptake and enzyme studies. J. Gen. Microbiol. 134,857–863.

[6] Kuenen, J.G. and Robertson, L.A. (1987) Ecology of nitrification and denitrification. In: *The Nitrogen and Sulphur Cycles* (Cole, J.A. and Ferguson, S., Eds.). Cambridge University Press. pp.162–218.

[7] Robertson, L.A., van Niel, E.W.J., Torremans, R.A.M. and Kuenen, J.G. (1988) Simultaneous nitrification and denitrification in aerobic chemostat cultures of *Thiosphaera pantotropha*. Appl. Env. Microbiol. 54,2812–2818.
[8] Beijerinck, W.M. (1910) Bildung und verbrauch von stickoxydul durch Bakterien. Centrallblat fur Bakteriol. (Abt. II) 25,30–63.
[9] Taylor, B.F. and Hoare, D.S. (1969) New facultative *Thiobacillus* and a reevaluation of the heterotrophic potential of *Thiobacillus novellus*. J. Bact. 100, 487–479.
[10] Vishniac, W. and Santer, M. (1957) The *Thiobacilli*. Bact. Rev. 21,195–213.
[11] Goa, J. (1953) A microbiuret method for protein determination; determination of total protein in cerebrospinal fluid. J. Clin. Lab. Invest. 5,218–222.
[12] Griess-Romijn van Eck (1966) Physiological and chemical tests for drinking water. NEN 1056, IV-2, Nederlands Normalisatie Instituut Rijswijk.
[13] Frear, D.S. and Burrell, R.C. (1955) Spectrophotometric method for determining hydroxylamine reductase activity in higher plants. Anal. Chem. 27,1664–1665.
[14] Fawcett, J.K. and Scott, J.E. (1960) A rapid and precise method for the determination of urea. J.Clin.Path. 13,156–159.
[15] Williams, P. (1984) Enzpack. A demonstration and calculation program for enzyme kinetics (BBC version). Elsevier Biosoft, Elsevier, Amsterdam.
[16] Muyzer, G., de Bruyn, J.C., Schmedding, D.J.M., Bos, P., Westbroek, P. and Kuenen, J.G. (1987) A combined immunofluorescence-DNA fluorescence staining technique for enumeration of *Thiobacillus ferrooxidans* in a population of acidophilic bacteria. Appl. Env. Microbiol. 53,660–664.
[17] Monod, J. (1942) Recherches sur la Croissance des Cultures Bacteriennes. Hermann, Paris.
[18] Heijnen, J.J. (1984) Biological industrial waste-water treatment minimizing biomass production and maximizing biomass concentration. PhD Thesis. Delft University of Technology, Delft, The Netherlands.
[19] Kucera, I., Boublikova, P. and Dadak, V. (1984) Function of terminal acceptors in the biosynthesis of denitrification pathway components in *Paracoccus denitrificans*. Folia Microbiol. 29,108–114.
[20] McCarthy, J.E. and Ferguson, S.J. (1981) Estimation with an ion-selective electrode of the membrane potential in cells of *Paracoccus denitrificans* from the uptake of the butyltriphenylphosphonium cation during aerobic and anaerobic respiration. Biochem. J. 196,311–321.
[21] Lam, Y. and Nicholas, D.J.D. (1969) Aerobic and anaerobic respiration in *Micrococcus denitrificans*. Biochim. Biophys. Acta 172,450–461.
[22] Alefounder, P.R., McCarthy, J.E.G. and Ferguson, S.J. (1981) The basis of the control of nitrate reduction by oxygen in *Paracoccus denitrificans*. FEMS Microbiol. Letts. 12,321–326.